# *Salmonella enteritidis* and other *Salmonella* in Laying Hens and Eggs from Flocks with *Salmonella* in their Environment

Cornelius Poppe, Roger P. Johnson, Christine M. Forsberg and Rebecca J. Irwin


## ABSTRACT

**Seven Canadian layer flocks with *Salmonella enteritidis* in their environment were investigated to determine the numbers of hens infected with *S. enteritidis*, the localization of *S. enteritidis* in organs of infected hens and the numbers of *S. enteritidis*-infected eggs produced by two affected flocks. By a microagglutination test (MAT) using *S. pullorum* antigens, these flocks had more seropositive hens (mean 51.9 ± 16.9%) than two *Salmonella*-free flocks (mean 13.0 ± 4.2%). Culture of tissues of 580 hens (433 seropositive) from the seven flocks detected 26 (4.5%) *S. enteritidis*-infected hens from two flocks. In one flock, 2/150 hens were infected with *S. enteritidis* phage type (PT) 8, which was confined to the ceca, and no *Salmonella* spp. were isolated from 2520 eggs (one day's lay). In the second flock, where 24/150 hens were infected with *S. enteritidis* PT13, extraintestinal infection was found in nine hens and involved the ovaries and/or oviduct in two hens. *Salmonella enteritidis* PT13 was isolated from one sample of egg contents and from one sample of cracked shells from among 14,040 eggs (one day's lay) from this flock. The overall prevalence of *S. enteritidis*-contaminated eggs from the two flocks with infected hens was less than 0.06%. Other *Salmonella* spp. isolated were *S. heidelberg* from 58 hens (10%), and *S. hadar*, *S. mbandaka* and *S. typhimurium* from one hen (0.2%) each. The MAT with antigens of *S. pullorum* had a sensitivity of 81% and a specificity of 24% for detecting *S. enteritidis*-infected hens. Selective enrichment on modified semisolid Rappaport-Vassiliadis agar was more reliable than tetrathionate brilliant green broth for isolation of *Salmonella* from samples of fecal origin, whereas the latter was superior for tissue samples.**

## RÉSUMÉ

**Les auteurs ont étudié sept élevages canadiens de poules pondeuses ayant *Salmonella enteritidis* comme agent infectieux dans leur environnement afin de déterminer le nombre de poules infectées avec *S. enteritidis*, la localisation de *S. enteritidis* dans les organes de poules infectées et le nombre d'œufs infectés par *S. enteritidis* produits par deux élevages affectés. Au moyen d'un test de microagglutination (MA) utilisant des antigènes de *S. pullorum*, ces élevages avaient plus de poules séropositives (moyenne 51,9 ± 16,9 %) que deux élevages exempts de *Salmonella* (moyenne 13,0 ± 4,2 %). La culture de tissus de 580 poules (433 séropositives) provenant des sept élevages permit de détecter 26 (4,5 %) poules infectées avec *S. enteritidis* dans deux élevages. Dans un des élevages, 2/150 poules étaient infectées avec le type phagique (TP) 8 de *S. enteritidis*, qui était limité au caeca, et aucun *Salmonella* spp. ne fut isolé de 2,520 œufs (ponte de une journée). Dans le deuxième élevage, 24/150 poules étaient infectées avec *S. enteritidis* TP13 et une infection extra-intestinale fut observée chez neuf poules et impliquait les ovaires et/ou l'oviducte chez deux poules. *Salmonella enteritidis* TP13 fut isolé à partir d'un échantillon de contenu d'œuf et d'un échantillon d'œuf à coquille craquée parmi les 14,040 œufs (ponte de un jour) provenant de cet élevage. Dans les deux élevages avec des poules infectées, la prévalence d'œufs contaminés par *S. enteritidis* était de moins de 0,06 %. Les autres *Salmonella* spp. isolés étaient *S. heidelberg* chez 58 poules (10 %), et *S. hadar*, *S. mbandaka* et *S. typhimurium* chez une poule chacun (0,2 %). Le test de MA avec les antigènes de *S. pullorum* avait une sensibilité de 81 % et une spécificité de 24 % pour détecter les poules infectées par *S. enteritidis*. L'enrichissement sélectif sur milieu gélosé semi-solide modifié de Rappaport-Vassiliadis était plus efficace que le bouillon tétrathionate au vert brillant pour l'isolement de *Salmonella* à partir d'échantillons d'origine fécale, alors que ce dernier était supérieur pour les échantillons de tissus. (*Traduit par Dr Serge Messier*)**


## INTRODUCTION

There has been a dramatic increase in human *Salmonella enteritidis* infections in the last five to ten years (1). In England and Wales, where *S. enteritidis* phage type (PT) 4 predominates, isolations of *S. enteritidis* from humans rose from 1087 in 1981 to 15,427 in 1988, and in 1988 accounted for 56% of all human *Salmonella* isolates (2,3). In the United States, the proportion of cases of human salmonellosis attributed to *S. enteritidis* increased from about 6% in 1980 to 20% (8340 cases) in 1989 (Annual Salmonella Report, 1990, United States Centers for Disease Control). Most isolates in the United States were identified as *S. enteritidis* PT8 (4,5). Phage type 8 also predominates in Canada, where over a similar period *S. enteritidis* has remained the third or fourth most common cause of human

Agriculture Canada, Health of Animals Laboratory, 110 Stone Road West, Guelph, Ontario N1G 3W4.

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salmonellosis, and in 1989 accounted for 9.1% of 8762 isolates (6).

Numerous outbreaks of *S. enteritidis* infections in humans have been associated with consumption of eggs or foods containing eggs (2,7–11). Egg contents may become contaminated with *Salmonella* from soiling of the shell with feces of infected hens, either by direct penetration of the shell or on breaking the eggs (12). There is evidence that *S. enteritidis* also gains access to egg contents by migrating from the cloaca to the reproductive organs, or transovarially following systemic infection and localization in the ovaries, oviducts or peritoneum of laying hens (13–15). Indeed, hens naturally infected with *S. enteritidis* have been found to carry the organism in the reproductive tract as well as in the ceca (13). Other *Salmonella* spp. including *S. pullorum*, *S. gallinarum*, *S. typhimurium*, *S. heidelberg*, *S. thompson* and *S. menston* have also been isolated from ovaries and oviducts of naturally infected hens (16–19).

The prevalence of *S. enteritidis* in eggs from infected flocks is quite variable (8,20–22). Some estimates were as high as 1% for conventional, caged flocks (23), and 50% for free-range home flocks (24). Much of this variation may be due to differences in invasiveness of the infecting strains, and hence the probability of localization in the reproductive tract. *Salmonella enteritidis* PT4 isolates were found to be more invasive for adult hens than most *S. enteritidis* PT8 strains, although one PT8 strain was equally invasive (15). In another study of orally infected and contact-exposed SPF hens, one PT13a was also highly invasive, and a high percentage of eggs produced in the first two weeks of infection contained *S. enteritidis* (25).

Although strains of *S. enteritidis* differ in virulence and invasiveness, naturally occurring infections in layer flocks are usually mild or subclinical, with little or no effect on egg production (26,27). Consequently infected flocks are difficult to identify. Among the procedures recommended to detect *S. enteritidis* in layer flocks are regular microbiological and/or serological testing (28,29). In order to respond promptly to the increasing public health risk, some agencies have recommended the well-established pullorum test for serodiagnosis of *S. enteritidis* infection (United Kingdom Ministry of Agriculture and Food, Statutory Instrument 1989, No. 1963; United States Department of Agriculture Federal Register Vol. 56, No. 20, January 1991, Part 87). The rationale for use of this test is the cross reactivity of antibodies to *S. enteritidis* with somatic antigens of standard and variant strains of *S. pullorum* (pullorum antigens). However, there is disagreement over the reliability of this test (28,30,31).

A recent nation-wide study to determine the prevalence of *S. enteritidis* and other *Salmonella* spp. in Canadian commercial egg producing flocks identified eight of 295 (2.7%) flocks with *S. enteritidis* in environmental samples (dust/fluff, eggbelt samples, feces) (32). Here we report follow-up investigations on these flocks with *S. enteritidis* in their environment. The main objectives of the study were to determine the presence and organ localization of *Salmonella* in birds from these flocks, to examine the prevalence of *S. enteritidis*-infected eggs in two flocks with hens infected with this serovar, and to evaluate pullorum antigens for serological detection of *S. enteritidis* infection. In addition, the modified semi-solid Rappaport-Vassiliadis (MSRV) agar plate (33) and tetrathionate brilliant green (TBG) broth methods of selective enrichment for *Salmonella* were compared.

## MATERIALS AND METHODS

### SAMPLING PROCEDURES

Of the eight layer flocks designated positive for *S. enteritidis* by environmental sampling in the initial survey (32), one was at the end of lay and was depopulated. The remaining seven flocks, designated 029, 074, 155, 161, 310, 379 and 400, were available for further study. They were located in various regions across Canada, and ranged in size from 666 to 41,661 birds. Two additional flocks, 228 and 229, free of *Salmonella* in the initial survey and designated negative flocks, were used as uninfected control flocks for serological studies.

Between 1.5 and five months after the initial survey, the farms were revisited for blood sampling. Approximately 300 birds in each positive flock were sampled. In the two negative flocks, 100 and 161 birds were bled. Cages within each barn were selected using a systematic random sampling plan, and one bird in each cage was wing-tagged and bled from the wing vein. Sera were tested by the microagglutination test (MAT) described below using pullorum antigens. For the purpose of necropsy examination and culture of selected tissues, 40–60 birds were chosen from each positive flock so that approximately 66% of the selected birds were seropositive. An additional 90 seropositive hens from flocks 161 and 310 were necropsied one month after the first group, and eggs were collected at the same time for microbiological testing. Environmental samples were collected from each flock on the day of necropsy, except for flocks 228 and 229 in which environmental samples had been collected at the time of blood sampling.

Necropsies were carried out at regional laboratories one to five weeks after blood sampling. Birds were asphyxiated in carbon dioxide, and cecal contents and samples of liver, spleen, pericardium, oviduct, and ovary were collected aseptically as individual specimens. As well, portions of these tissues from each bird were pooled to one sample per bird. The samples, in sealed containers with icepacks, were shipped by air or road to the Guelph laboratory.

As in the initial survey (32), environmental samples from each flock consisted of 60 randomly collected fecal droppings pooled into 20 groups of three, and 12 dust/fluff samples from eggbelts (where present) or from vents, fans and walls, pooled into four groups of three. All samples were collected with sterile materials and shipped under ice by air or road to the Guelph laboratory.

All eggs from one day's lay in flocks 161 (2520 eggs) and 310 (14,040 eggs) were collected unwashed, boxed and transported directly from the farms to the Guelph laboratory in a refrigerated truck. At the laboratory, the eggs were stored in their original boxes in a cold-room at 8°C for a maximum of 45 days before culture.

SEROLOGICAL TESTS

Sera were tested by a MAT similar to that described by Williams and Whittemore (34). Standardized unstained whole cell antigens of standard and variant strains of *S. pullorum* were supplied by the Animal Diseases Research Institute of Agriculture Canada, Nepean, Ontario. For each antigen, sera were diluted 1:10 in 25 μL volumes in duplicate wells of U-bottomed microtiter plates. The diluent was phosphate buffered saline (PBS) pH 7.4 containing 0.005% safranin. Stock antigen was diluted in 0.5% phenol-saline to an optical density equal to four times McFarland's tube no. 1, and 25 μL were added to each test well, giving a final serum dilution of 1:20 and a final antigen concentration equivalent to two times McFarland's tube no. 1. Known positive and negative serum controls were included with each batch of test sera. Plates were read after incubation for 18–24 h at 37°C. Results were recorded as positive (> 50% agglutination), suspicious (< 50% agglutination) or negative (no agglutination). Birds testing positive with either standard or variant antigen were scored as seropositive, and those with suspicious or negative results were grouped as seronegative.

MICROBIOLOGICAL PROCEDURES

Isolation of *Salmonella* was attempted from all environmental samples and all pooled tissue samples. Except from flock 029, the first flock studied, individual tissue samples were processed only when the pooled sample from one or more birds in the flock was positive for *S. enteritidis*. Samples were cultured by preenrichment then selective enrichment as described previously (32). Briefly, samples were agitated in a Stomacher in nine times their weight of buffered peptone water (BPW) (Difco) in their original plastic bags and incubated at 37°C for 18–24 h. For selective enrichment, 0.1 mL of the preenriched BPW was inoculated onto the periphery of a modified semisolid Rappaport-Vassiliadis (MSRV) agar plate (33), and incubated at 42°C for 18–24 h. Cultures migrating more than 20 mm into the MSRV were streaked onto MacConkey (MC) agar (Difco). The MSRV plates were scored as negative and discarded if no migratory growth was observed after incubation for 72 h.

For comparison, selective enrichment was also carried out using tetrathionate brilliant green (TBG) broth. One mL of the preenriched BPW was inoculated into 9 mL of TBG broth (Difco) which was incubated for 24 h at 43°C then streaked onto brilliant green sulfa (BGS) agar (Difco). Typical *Salmonella* colonies were subcultured onto Luria Bertani (LB) agar plates (35).

Eggs were first candled to sort cracked from intact eggs. Intact eggs were surface sterilized by immersion in 70% alcohol for 2 min, air-dried in a fume hood for 10 min then cracked with a sterile knife. Shells were discarded and egg contents from five eggs were pooled and mixed in a Stomacher for 1 min in twice their volume (approximately 500 mL) of BPW (20). After overnight incubation at 37°C, the preenrichment broths were cultured by both the MSRV and TBG selective enrichment methods described above, except that TBG broth cultures were streaked onto bismuth sulphite (BS) agar (Difco) as well as BGS agar. Cracked eggs were used for shell culture only. The surface of the eggs was not sterilized before cracking. Culture methods were the same as for intact eggs except that shells from four eggs were pooled and macerated in BPW in a blender jar instead of a Stomacher.

IDENTIFICATION OF ISOLATES

Up to five colonies were picked from MC and LB agar plates and tested by slide agglutination tests using *Salmonella* polyvalent O antiserum (Bacto *Salmonella* O antiserum poly A-I and Vi) (Difco). Positive isolates were tested similarly with *Salmonella* O group $D_1$ antisera (Bacto *Salmonella* O antiserum group $D_1$ factors 1, 9, 12; Difco) to determine if they could be *S. enteritidis* strains. All putative *Salmonella* isolates were also examined for susceptibility to lysis by a mixture of polyvalent *Salmonella* phage O-1 and a bacteriophage specific for the O groups $E_1$–$E_4$ (36,37).

To minimize the number of isolates of *Salmonella* to be serotyped and biotyped, the plasmid profiles of all isolates testing positive in the above procedures were determined as described previously (32). All isolates from one flock were tested on one gel for ease of comparison. Isolates representing different plasmid profiles were then biotyped and serotyped. Thirty biochemical tests were performed on each isolate using the Gram-Negative Idenfication (GNI) card with the Vitek Automated Microbiology System (Vitek Systems, Hazelwood, Missouri). Procedures for serotyping of *Salmonella* isolates have been described previously (38,39).

Isolates identified as *S. enteritidis* strains were typed with phages obtained from the Division of Enteric Pathogens, Central Public Health Laboratory, London, England (40).

## RESULTS

SEROLOGICAL TESTING

Overall rates of seropositivity of 248–299 sera from the seven positive flocks against either variant or standard strains of *S. pullorum* ranged from 29–77% (Table I). Substantially more birds were seropositive in flocks 161 and 310 in which infected birds were subsequently identified. Also there were marked differences between flocks in the results for each antigen. For example in flock 310, 74% of sera reacted with the variant strain and 31% with the standard strain, while in flock 379, seroreactors with the standard strain were more numerous (25% vs 9%). Less than 20% of sera gave suspicious reactions, and negative results were obtained for 20% (flock 310) to 50% (flock 379) of the birds tested (data not shown). In the two negative flocks, 10% and 16% of birds were seropositive (average 13%), with most being positive for only one of the two pullorum antigens (Table I).

ISOLATION OF *S. ENTERITIDIS*

No *Salmonella* were isolated from environmental samples from flocks 228 and 229. Follow-up environmental samples from the seven flocks previously positive for *S. enteritidis* contained *Salmonella*, and *S. enteritidis* was again isolated from four flocks (Table I). The highest numbers of *S. enteritidis*-positive environmental samples were from flock 161 (9/20 pooled fecal samples, 4/4 pooled dust/fluff samples) and flock 310 (7/20

**TABLE I. Follow-up serological and microbiological investigations in seven layer flocks with *S. enteritidis* in their environment**

| Flock (No. of hens) | *Salmonella* isolated from the environment | | Percentage[a] of birds seropositive | | | Results from necropsy | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial[b] | Follow-up | V | S | V or S | Total No. | No. sero-positive | No. culture-positive for SE[c] | No. culture-positive for other *Salmonella* |
| 029 (666) | *S. enteritidis* | *S. typhimurium* *S. schwarzengrund* | 41 | 32 | 52 | 40 | 25 | 0 | 1 (*S. hadar*) |
| 074 (41,661) | *S. enteritidis* *S. agona* *S. hadar* | *S. enteritidis* | 22 | 45 | 53 | 60 | 38 | 0 | 0 |
| 155 (10,200) | *S. enteritidis* | *S. enteritidis* | 28 | 33 | 46 | 60 | 40 | 0 | 0 |
| 161 (3,832) | *S. enteritidis* | *S. enteritidis* *S. agona* | 43 | 59 | 69 | 150 | 130 | 2 | 0 |
| 310 (17,740) | *S. enteritidis* | *S. enteritidis* | 74 | 31 | 77 | 150 | 128 | 24 | 1 (*S. mbandaka*) |
| 379 (40,434) | *S. enteritidis* | *S. heidelberg* | 9 | 25 | 29 | 60 | 41 | 0 | 10 (*S. heidelberg*) |
| 400 (15,800) | *S. enteritidis* *S. heidelberg* | *S. heidelberg* *S. typhimurium* | 17 | 29 | 37 | 60 | 41 | 0 | 48 (*S. heidelberg*) 1 (*S. typhimurium*) |
| 228 (7,200) | none | none | 3 | 7 | 10 | not tested | | not tested | |
| 229 (5,543) | none | none | 9 | 9 | 16 | not tested | | not tested | |

[a] Percentage of 248–299 birds (flocks 029, 074, 155, 161, 310, 379, 400), 100 birds (flock 228) and 161 birds (flock 229) showing < 50% agglutination in MATs against Variant (V), Standard (S) or either strain (V or S) of *S. pullorum*
[b] From reference 32
[c] *S. enteritidis*

pooled fecal samples, 2/4 pooled dust/fluff samples). Environmental isolates in each flock were recovered from fecal samples, while dust/fluff samples were culture-positive in only three flocks. However, proportionally more dust/fluff samples than fecal samples were positive (2–4/4 dust/fluff vs 1–9/20 fecal).

Culture of pooled tissues from 580 necropsied hens from the seven flocks detected 26 *S. enteritidis*-infected birds; two from flock 161 and 24 from flock 310. When individual tissue samples of the 150 necropsied hens of each of these flocks were cultured, *S. enteritidis* was recovered only from tissues of 19 of the 26 birds with positive pooled samples. Isolations were made most frequently from cecal contents, which were positive in 18/19 birds, and were the only positive samples in ten hens. Infection of extra-intestinal organs was detected in nine hens, and involved ovary and/or oviduct in two hens. Other tissues involved were spleen, seven hens; liver, five hens; and pericardial sac, two hens. All isolates of *S. enteritidis* from each affected flock displayed the same plasmid profiles and were of the same phage type. Tissue and environmental isolates from flocks 161 and 310 were PT8 and PT13, respectively, while environmental isolates from flock 074 were PT13a, and those from flock 155 were PT8.

### ISOLATION OF OTHER *SALMONELLA* SEROVARS

Other *Salmonella* serovars isolated from environmental samples were *S. heidelberg* and *S. typhimurium* from two flocks, and *S. schwarzengrund* and *S. agona* from one flock each (Table I). Culture of pooled tissue samples revealed that *S. heidelberg* was the most common serovar isolated from the birds in this study. It was recovered from 10/60 pooled samples of flock 379, and from 48/60 pooled samples of flock 400, giving an overall prevalence of 10% (58/580). Other serovars isolated from tissues were much less prevalent: *S. typhimurium* was cultured from 1/60 pooled samples from flock 400, *S. hadar* from the pericardial sac of one hen in flock 029, and *S. mbandaka* was isolated from the ovary of one hen from flock 310.

### CULTURE OF EGGS

For flock 161, none of 2520 eggs tested as 488 pooled samples of egg contents and 23 pooled samples of shells yielded *Salmonella*. For flock 310, 14,040 eggs were tested as 2675 pooled samples of egg contents and 115 pooled samples of cracked shells. *Salmonella enteritidis* was isolated from one pooled sample of egg contents and one pooled sample of shells, by both TBG and MSRV selective enrichment methods. *Salmonella thompson* was isolated from one other pooled sample of egg contents. Due to pooling, a positive sample of egg contents represented from one to five infected eggs, and a positive sample of egg shells represented from one to four infected eggs. Based on these ranges the overall prevalence of *S. enteritidis*-infected eggs in the two flocks was 0.012–0.054%.

### COMPARISON OF SELECTIVE ENRICHMENT METHODS

Of a total of 123 isolations of *Salmonella*, 116 (94%) were made from TBG broth and 95 (77%) from MSRV agar (Table II). Eighty-eight isolates (72%) were recovered by both methods of selective enrichment. This slightly higher isolation rate with TBG broth reflected the results obtained with pooled tissue samples, which accounted for 86/123 isolations. Differences were more marked however when results for individual tissues were compared. The MSRV method was superior to TBG broth for isolation

from cecal contents (18/18 isolates vs 14/18 isolates). In contrast, all 19 isolates from extraintestinal organs (liver, spleen, pericardial sac, ovary, oviduct) were recovered from TBG broth, compared to 5/19 (26%) on MSRV agar.

EVALUATION OF THE MICROAGGLUTINATION TEST

The mean rate of seropositivity for the seven positive flocks (51.9 ± 16.9%) was considerably higher than for the two negative flocks (13.0 ± 4.2%). Also the two flocks with *S. enteritidis*-infected hens had higher rates of seropositivity than the five other flocks (mean values 73.0 ± 5.7% vs 43.4 ± 10.3%).

Serological and microbiological results for necropsied birds were used to evaluate the MAT using pullorum antigens as an indicator of infection in individual birds. As shown in Table I, the proportion of necropsied hens that were seropositive in each flock ranged from 25/40 (63%) in flock 029 to 130/150 (87%) in flock 161. Overall, 443/580 (76%) necropsied birds were seropositive. Among the 26 birds infected with *S. enteritidis*, 21 were seropositive, giving a sensitivity of 81%. Of the 554 uninfected hens, 132 were seronegative, resulting in a specificity of 24%. The predictive value of a positive MAT result was 5%.

**TABLE II. Comparison of modified semisolid Rappaport-Vassiliadis (MSRV) agar and tetrathionate brilliant green (TBG) broth as selective enrichment media for isolation of *Salmonella* spp. from necropsy samples from laying hens**

| Sample | No. positive for *Salmonella* /No. tested | No. of *Salmonella* isolations made in: MSRV agar | TBG broth | MSRV and TBG |
|---|---|---|---|---|
| Cecal contents | 18[a]/340 | 18 | 14 | 14 |
| Liver | 5[a]/340 | 3 | 5 | 3 |
| Spleen | 7[a]/340 | 1 | 7 | 1 |
| Pericardial sac | 3[b]/340 | 1 | 3 | 1 |
| Ovary | 2[c]/340 | 0 | 2 | 0 |
| Oviduct | 2[a]/340 | 0 | 2 | 0 |
| Pool of above | 86[d]/580 | 72 | 83 | 69 |
| Total | 123/2620 | 95 | 116 | 88 |
| (%) | (4.7) | (77.2) | (94.3) | (71.6) |

[a] All *S. enteritidis* isolates
[b] 2 *S. enteritidis* isolates, 1 *S. hadar* isolate
[c] 1 *S. enteritidis* isolate, 1 *S. mbandaka* isolate
[d] 58 *S. heidelberg*, 26 *S. enteritidis*, 1 *S. hadar*, 1 *S. typhimurium* isolates

## DISCUSSION

Selection procedures for this study were designed to favor detection of *S. enteritidis* infection. *Salmonella enteritidis* had been isolated from environmental samples of all flocks in the initial survey, and more than 60% of necropsied birds from each flock were seropositive with standard or variant strains of the antigenically related organism, *S. pullorum*. Culture of pooled tissue samples from 580 hens from these flocks detected a total of 26 (4.5%) *S. enteritidis*-infected hens in two flocks. All isolates of *S. enteritidis* from each infected flock were of the same phage type, namely PT8 in flock 161 and PT13 in flock 310. The numbers of *S. enteritidis*-infected birds within these two flocks were 2/150 (1.3%) in flock 161 and 24/150 (16%) in flock 310, and less than 0.06% of eggs from these flocks were contaminated with *S. enteritidis*. These findings are substantially lower than usually found in flocks in the United Kingdom, where *S. enteritidis* PT4 predominates (23,28,30).

Results of culture of individual organs of birds from the two *S. enteritidis*-infected flocks (161 and 310) indicated that the ceca were the predominant site of *S. enteritidis* infection. Localization in extraintestinal organs was not detected in hens from flock 161, and occurred in less than 40% of infected hens in flock 310. Ovaries or oviduct were infected in only 2/150 hens in flock 310. Others investigating layer flocks naturally infected with *S. enteritidis* have found more frequent involvement of reproductive organs, and also report higher prevalences of *S. enteritidis*-infected eggs, presumably due to transovarian infection (20,23,41). Those flocks were infected with *S. enteritidis* PT4 strains, which in comparative studies were more invasive than most PT8 strains (15). Our findings suggest that the strains isolated from the two Canadian flocks, particularly the PT8 strain of *S. enteritidis* from flock 161, were less invasive, and were less likely to be transmitted transovarially to eggs.

Our estimate of less than 0.06% for the prevalence of *S. enteritidis*-infected eggs from infected flocks should be interpreted conservatively. Firstly, it is based on only two isolations from pooled samples of eggs from two flocks. With so few infected eggs, and the low numbers of organisms found in eggs, it is difficult to trace *S. enteritidis* and establish a true prevalence (*Salmonella enteritidis*: An Appraisal of its Association with Eggs and Foodborne Illness. Report for the Canadian Egg Marketing Board, M.E. Stiles, 1990). Hens in these two flocks were more than 30 weeks old, and may have been less likely to lay infected eggs than younger flocks (27). In addition, many factors including route of infection, storage temperature and handling may influence the survival and growth of *S. enteritidis* in eggs (9,42,43). For example, storage of eggs at 8°C, as in this study, prevents multiplication of the organism (42,44). It should be noted that the tested eggs were not washed or sanitized before shipping. Consequently isolates, especially those from shell samples of cracked eggs, may have been present due to fecal contamination of the shell.

Our previous survey of *Salmonella* spp. in environmental samples from Canadian layer flocks showed that *S. heidelberg* was more prevalent than any other serovar (32). Similar results were obtained in the present study. Infecting 10% of all necropsied hens, *S. heidelberg* was again the most common serovar, and was twice as common as *S. enteritidis*. In one flock 80% of hens were infected. The reasons why this serovar is so prevalent are not known, although prolonged, extensive colonization of the ceca of chickens may prevent infection with antigenically related serovars (45). Other possible explanations include more frequent egg shell penetration by *S. heidelberg*,

resulting in a higher frequency of infection in day-old chicks and hence adult hens, or greater invasiveness, leading to a higher frequency of transovarian infection (17). Although we did not culture individual organs from birds in the two affected flocks, others found that *S. heidelberg* was the most common serovar isolated from ovaries of laying hens at slaughter (46). In Canada, *S. heidelberg* is isolated from humans at approximately the same frequency as *S. enteritidis* (6), and while a relationship between *S. heidelberg* in laying hens and human salmonellosis has not been established, eggs have been incriminated as the cause of a large outbreak of *S. heidelberg* infection in people in New Mexico (47).

The use of environmental sampling, two methods of selective enrichment and serological testing with pullorum antigens allowed some assessment of these methods for detection of *Salmonella*, and particularly *S. enteritidis* in laying hens. Detection of *S. enteritidis*-infected birds in only two of seven flocks with *S. enteritidis* in their environment in the initial survey raised the question of the predictive value of environmental sampling. While this may have been due in part to intervals of up to five months between initial and follow-up studies, intervals for the two flocks with *S. enteritidis*-infected hens were longer than for most of the other flocks. Furthermore, infections of adult hens with *S. enteritidis* and other *Salmonella* frequently persist for more than 22 weeks (25). Nevertheless, samples collected at the time of necropsy were more reliable, revealing *S. enteritidis* in the environment of four rather than seven flocks, and the greatest numbers of positive environmental samples were from the two flocks with infected hens. In addition, three of the four other serovars isolated from necropsied birds were also present in environmental samples. Perhaps more importantly, *S. enteritidis* was not isolated from birds in flocks in which environmental samples were negative for this serovar. These results, and the finding that all environmental and tissue isolates from each flock were of the same plasmid profile and phage type, favor rather than deny the usefulness of environmental sampling as a screening procedure.

In comparing the two methods of selective enrichment for *Salmonella*, we found that the MSRV method was superior to TBG broth for samples of cecal contents, but was considerably less sensitive for culture of extraintestinal tissues. These results suggest that the type of sample should be considered in choosing methods of selective enrichment. From our results, and previous studies (48,49) the MSRV method appears to be the most suitable for isolation of *Salmonella* from feces and samples contaminated with feces, such as dust, feathers, litter and food. For samples of tissue origin, TBG broth would be more reliable.

Agglutination tests employing antigens of *S. pullorum* to detect *S. enteritidis* infection have been advocated because the two organisms share the somatic antigens O:9 and O:12 (50). While some reports indicate that these tests are insensitive (30,31), others have found that the sensitivity is adequate for field testing (28). In the present study, flocks with environmental evidence of exposure to *S. enteritidis* had much higher rates of seropositivity than *Salmonella*-free flocks, and 21/26 (81%) *S. enteritidis*-infected hens tested positive. While these results indicate the MAT was reasonably sensitive, its specificity (24%) and hence predictive value (5%) was very low. This lack of specificity was not unexpected, since O12, the dominant somatic antigen of *S. enteritidis* (28), is also carried by many of the most prevalent *Salmonella* serovars in layer flocks, including *S. typhimurium*, *S. heidelberg*, *S. schwarzengrund* and *S. agona*, which were isolated from environmental and in some cases tissue samples from five of the seven *S. enteritidis*-positive flocks. These serovars may have been partially responsible for the high rates of seropositivity in the flocks with *S. enteritidis* in the environment compared to the two *Salmonella*-free flocks. Consequently pullorum antigens appear to be unreliable for screening flocks or individual hens for specific serological evidence of *S. enteritidis* infection.

The results of this study extend our knowledge of the prevalence of *S. enteritidis* in Canadian laying flocks. In our previous survey based on environmental sampling (32), 8/295 (2.7%) Canadian laying flocks had evidence of *S. enteritidis* infection. From results of the present study, 2/7 of these flocks contained *S. enteritidis*-infected hens, indicating an overall prevalence of less than 1% for infected flocks. Less than 5% of necropsied hens from these two flocks were infected with this serovar. Infecting strains were of the same phage type as identified in environmental samples, namely PT8 in one flock and PT13 in the other. Extraintestinal infections were present only in the flock infected with *S. enteritidis* PT13, and less than 0.06% of unwashed, unsanitized eggs from this flock contained in *S. enteritidis*. As in the initial survey, *S. heidelberg* was the most prevalent *Salmonella* serovar.

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